Dog ´skin parasite load, TLR-2, IL-10 and TNF-α expression and infectiousness

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Effect of skin parasite load on TLR-2, IL-10 and TNF-α expression and infectiousness to sandflies during canine leishmaniosis

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DISCLOSURES

None.

ABSTRACT

Visceral leishmaniosis is a zoonotic disease that is transmitted by *Lutzomyia longipalpis* sandflies. Dogs are the main peri-urban reservoir of the disease, and progression of canine leishmaniosis is dependent on the type of immune response elaborated against the parasite. Type 1 immunity is characterized by effective cellular response, with production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α). In contrast, Type 2 immunity is predominantly humoral, associated with progression of the disease, and mediated by anti-inflammatory cytokines such as interleukin 10 (IL-10). Although seemly important in the dynamics of leishmaniosis, other gene products such as toll-like receptor 2 (TRL-2) and inducible nitric oxide synthase (iNOS) exert unclear roles in the determination of the type of immune response. Given that the dog skin serves as a micro-environment for the multiplication of *Leishmania spp.*, we investigated the parasite load and the expression of TLR-2, iNOS, IL-10 and TNF-α in the skin of 29 infected and eight control dogs. We found that increased parasite load leads to up-regulation of TLR-2, IL-10 and TNF-α, indicating that abundance of these transcripts is associated with infection. We also performed a xenodiagnosis to demonstrate that increased parasitism is a risk factor for infectiousness to sandflies.

INTRODUCTION

The domestic dog (*Canis lupus familiaris*) is the main peri-urban reservoir of leishmaniosis in Brazil[1]. This zoonotic disease is caused by *Leishmania infantum* (Syn. c*hagasi*), a protozoon transmitted to vertebrate hosts by female sandflies from the *Lutzomyia longipalpis* species[2]. The progression and severity of canine leishmaniosis (CanL) has been argued to relate both to the infectivity of the parasite and the immunogenetic profile of the host[3]. Dogs that do not develop clinical disease usually present an effective cellular response characterized by Type 1 immunity, which is presumably mediated by pro-inflammatory cytokines such as interleukin 2 (IL-2), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α)[4,5]. On the other hand, sick dogs often exhibit predominance of a Type 2 profile, with expression of transforming growth factor beta (TGF-β) and interleukin 10 (IL-10), as well as an exacerbated humoral immune response due to B lymphocyte proliferation[6].

The activation of Toll-like receptors has recently emerged as an important step in determining the type of immune response[7,8,9] and therefore may have an impact on the development of the disease[10,11]. Toll-like receptors are capable of recognizing lipophosphoglycan molecules present in the surface of the flagellated promastigote form of the protozoon[12], resulting in intracellular reactions responsible for the production of pro-inflammatory and anti-inflammatory cytokines[13,14,15]. In particular, increased expression of Toll-like receptor 2 (TLR-2) has been related to a lower parasite load in the skin of naturally infected dogs, as well as to reduced infectiousness to the vector[16]. Of note, TLR-2 can affect the production of the inducible nitric oxide synthase (iNOS) enzyme, which catalyzes nitric oxide (NO) production[17,18,19,20,21]. Conversely, TLR-2-deficient mice infected with *Leishmania donovani* showed enhanced macrophage antileishmanial activity with no changes in the liver expression of iNOS as compared to wild type mice[22].

Considering the importance of the skin in the transmission of parasites of the *Leishmania* genus, we aimed at evaluating the expression of the immune response genes TLR-2, iNOS, TNF-α and IL-10 in the skin of dogs naturally affected by CanL and relate them to the skin parasite load (PL). We also investigated the relationship between skin PL and infectiousness to sandflies in a xenodiagnosis.

MATERIAL AND METHODS

Ethics statement

This research was approved by the local Ethics and Animal Welfare Committee (CEUA-FOA/FMVZ, process 01984-2012).

Study area

Araçatuba (latitude 21°2'3"S; longitude 50°5'58"W) is located in the Northwestern region of the state of São Paulo (Brazil), and it is an endemic area for leishmaniosis since 1999. The majority of the cases take the visceral form, whereas cutaneous leishmaniosis is sporadic in this area. A seroepidemiological survey is conducted annually by the Municipal Center for Zoonosis Control (CZC), and dogs that present positive serological diagnosis or amastigotes in a lymph node biopsy are recruited and culled after the consent of owners to comply with the local legislation.

Animals

Mongrel dogs from the CZC aging from 1 to 10 years were initially divided into two categories: (i) the control group (C), comprising eight dogs from an adoption program that were negative for both the serological test and the parasite quantification in blood and lymph node aspirates by quantitative polymerase chain reaction (qPCR); and the parasited group (P), including 29 naturally infected dogs

with positive serological and skin qPCR results. All dogs in group P presented clinical alterations compatible with CanL, including: onychogryphosis (n=25); dermatopathies as dermatitis, alopecia, hyperkeratosis or ulcerated lesions (n=24); lymphadenopathy (n=22); ocular lesions such as conjunctivitis, keratoconjunctivitis or uveitis (n=18); and cachexia (n=3). These animals were further classified as stage I, II or III according to Solano-Galego and collaborators[23].

Diagnosis of CanL

Anti-Leishmania antibodies were evaluated in serum samples by the TR-DPP® rapid test and confirmed by EIE-ELISA® (Bio-Manguinhos/Fiocruz-Rio de Janeiro). All samples were processed in duplicates following the manufacturer's instructions. Ear tip biopsies of uninjured skin of positive and negative dogs were taken with a 3 mm punch to assess the parasite load and the gene expression of TLR-2, iNOS, TNF-α and IL-10. These biopsies were taken immediately after euthanasia, following current legislation. All samples were stored at -80 °C in RNAlater-ICE (Ambion AM7030 - Life Technologies®/USA) until processing. The absence of infection in dogs serologically negative for CanL (controls) was confirmed in blood and lymph node samples by qPCR using primers described by Rodgers et al[24]. DNA was extracted using the QIAamp DNA mini kit® (Qiagen, Hilden/Germany), according to the manufacturer's instructions. DNA was eluted in a final volume of 50 µL for blood samples and 40 µL for lymph node samples. These were evaluated in duplicate by qPCR according to using 40 ng of DNA. The reaction efficiency values, the coefficient of determination (r^2) and the slope (β) were assessed using a 10 fold serial dilution of *L. infantum* DNA (L579 MHOM/BR/1974/PP75).

Skin parasite load

DNA from skin samples was extracted using the Ear Tissue-ET kit (Chemagen cmg-1011, Perkin Elmer®), according to the manufacturer's instructions. The amplification of a 120 bp minicircle kinetoplast DNA (kDNA) fragment of *Leishmania spp*. was carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems®). Primers Leish1 (AACTTTTCTggTCCTCCgggTAg) and Leish2 (ACCCCCAgTTTTCCgCC) described by Francino et al.[25] and modified by Calvo-Bado (unpublished) were used in a concentration of 900 nM each. Additionally, 250 nM of the probe 6FAM 5' AAAAATgggTgCAgAAACCCCgTTC-3' – BBQ was used. The reaction was carried out in a total volume of 15 µL containing 2x TaqMan master mix (Applied Biosystems®). Amplification conditions were 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Quantification of PL was performed by comparing the sample exponential phase threshold (Ct - cycle threshold) values with those obtained from a standard curve constructed from 10-fold serial dilutions (0.01 to 105 parasites/ml) of DNA extracted from cultured *L. infantum* (ITAMP DD8-263) promastigotes. All samples were assessed in triplicates.

Gene expression in the skin

Total RNA was extracted from skin samples and stored at -80 °C in RNAlater-ICE (Ambion AM7030-Life Technologies®/USA) using the RNeasy Mini Kit (74104-Qiagen®), according to the manufacturer's specifications. Total RNA was quantified by a NanoDrop® spectrophotometer ND-100 and used conditional on a 260/280 ratio between 2.0 and 2.3. For cDNA production, 200 ng of each RNA sample was subjected to reverse transcription using the QuantiTect Reverse Transcription commercial kit (205311-Qiagen®, Hilden/Germany), according to the manufacturer's specifications. Canine-specific primers for amplifying fragments of cDNA from TLR-2, iNOS, IL-10 and TNF-α, as well as from the reference gene hipoxanthine phosphoribosyltransferase 1 (HPRT-1), were selected from the literature (**Table 1**). The qPCR assay was carried out using SsoFastTM EvaGreen Supermix (Bio-Rad Laboratories®-CA/USA), 320 nM of each primer and 1 µL of cDNA in a total volume of 25 µL. Duplicates were incubated at 95 °C for 30 seconds, followed by 45 cycles of 95 °C for 5 seconds and 60 °C for 10 seconds, when the fluorescence data were captured, followed by dissociation curve in

65 °C to 95 °C, with an increase of 0.5 °C every 10 seconds. For each evaluated gene, values of the reaction efficiency, r^2 and β were obtained from the amplification of a serial dilution of a cDNA pool from both groups C and P (**Table 1**). Quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method[26], using HPRT-1 as a reference gene. The results were expressed as the relative gene expression that indicated how many times (fold change) the cytokine gene expression was higher or lower in the infected dogs in relation to the control group. The gene expression of TLR-2, IL-10 and TNF-α was evaluated in 29 dogs and the expression of iNOS was evaluated only in 24 infected dogs, apart from eight control dogs.

Xenodiagnosis

Adult sandflies were field-caught by manual aspiration in urban areas of Araçatuba. To facilitate capture, collections were carried out in households sheltering at least one chicken, as the latter favors the aggregation of phlebotomines[2]. Sandflies were transferred to 20 x 20 cm fine-mesh cages and kept at 86% humidity and 25.5 °C in a climate-controlled unit (BOD - Electro lab - 101 M). Sandflies were maintained on a diet of honey solution (1:1 in distilled water) for 12 hours before being separated into tested and control groups. At 12 hours, to enhance the chance of sandflies feeding during the xenodiagnosis, only non-blood-fed and partially fed (maximum half of the midgut) female field-caught sandflies were separated for blood feeding on a dog from group P (xenodiagnosis). Engorged field-caught individuals were reserved as naturally infected specimens that would display only natural infection (control group of sandflies), and were offered only honey solution. An equal number of males was also separated into the cages in order to maximize normal behavior and survival. Following xenodiagnosis, sandflies were recovered and both the tested and control groups were maintained for five days under the aforementioned conditions. Each infected dog was restrained in a metal cage, covered by another fine-mesh net for 12 hours (covering periods of crepuscular sandfly activity), with an average of 30 *L. longipalpis* females and accompanying males. A total of 29 xenodiganoses were performed between November 2012 and May 2014. No xenodiagnosis was performed on negative dogs (n=8) due to the fact that female sandflies were field-caught and could transmit the parasite to healthy dogs. Due to variations on density of sandflies during captures, we analyzed data of a subset of 18 xenodiagnosis, for which the average number of control and tested sandflies were 23.4 ± 15.2 (min: 2 / max: 50) and 27.4 ± 9.4 (min: 13 / max: 47), respectively.

Parasite load in sandflies

DNA extraction was performed in individual sandflies using the protocol of Kato et al.[27] with modifications: dissected sandflies were centrifuged for 2 minutes at 15871 g and macerated with a pestle and glass beads (2 mm). After discarding the supernatant, 75 µL of the lysis buffer solution (150 mM NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.1% SDS and 20 µL of proteinase K - Ambion®-D10860413) were added, followed by incubation for 6 hours at 65˚C. Samples were then centrifuged for 2 minutes at 15871 g, and 35 µL sterile water added and mixed thoroughly, followed by further centrifugation (15871 g for 2 minutes). The supernatant containing DNA was transferred to another tube. The qPCR procedure was performed as described for the dog skin. Sandflies were then classified as “infected” if their PL were different from zero and as “non-infected” otherwise for part of the downstream analyses. All samples were assessed in triplicates.

Statistical analysis

In order to assess the influence of increased skin parasitism on gene expression, dogs from group P were further divided into two subgroups based on a median split on PL values. Therefore, animals were allocated to one of the following three groups for statistical analysis: C = eight control uninfected dogs, as defined earlier; MP = 11 dogs from group P with PL values between 2.19 x 10^2 and 2.27 x 10^5 parasites/mL ; and HP = 18 dogs from group P with PL values between 2.27 x 10^5 and 4.13 x 10^7

parasites/mL. Distribution of PL values according to group is displayed in **Supplementary Figure 1**. An analysis of variance (ANOVA) with a *post hoc* Tukey's Honest Significant Difference (HSD) test was used to assess differences in -ΔCt between groups. For the genes exhibiting significant differences ($p < 0.05$) among groups, we merged MP and HP back to a single parasited group (P) in order to examine the fold change in expression relative to group C using the $2^{-\Delta\Delta Ct}$ method[26]. We also applied logistic regression models to the xenodiagnosis data considering a binary response variable encoded as 1 = "infected sandfly" and 0 = "non-infected sandfly", according to qPCR results. In the first model, we examined the contribution of exposure to infected dogs to the probability of sandfly infection by including an indicator variable assuming values 0 and 1 for control and tested sandflies, respectively. Additionally, we performed a permutation test for differences in prevalence between control and tested sandflies with 10^{-6} randomizations of the data. Then, we used data only on tested sandflies to fit skin PL as a covariate in the logistic model to assess the influence of the level of skin parasitism on the probability of sandflies being infected. Prior to this analysis, skin PL was transformed to a $\log_{10}(1 + PL)$ scale. Finally, we compared the ranks of parasite load of sandflies fed on HP dogs with those that were fed on MP dogs with a Mann-Whitney U test to evaluate whether parasite load in phlebotomines was dependent on parasite load in dogs. All analyses were performed in R v3.3.0 (available at: https://www.r-project.org/).

RESULTS

Increased load of *Leishmania* leads to up-regulation of IL-10, TNF-α and TLR-2 in the dog skin

We used ANOVA with Tukey's HSD to compare the skin relative expression of iNOS, IL-10, TNF-α and TLR-2 among three groups of dogs (see details in Material and Methods): uninfected controls (group C, n = 8), animals with low-to-moderate infection (group MP, n = 11) and animals with moderate-to-high infection (HP, n = 18). Expression levels differed between groups only for IL-10 ($p_{ANOVA} = 0.008$), TNF-α ($p_{ANOVA} = 0.007$) and TRL-2 ($p_{ANOVA} = 5.8 \times 10^{-6}$), with up-regulation of these transcripts following increased PL (**Figure 1**). Tukey's HSD was only significant ($p_{HSD} < 0.05$) in the comparisons of groups MP-HP for IL-10 ($p_{HSD} = 0.008$), C-HP for TNF-α ($p_{HSD} = 0.009$), and C-HP ($p_{HSD} = 6.6 \times 10^{-5}$) and MP-HP ($p_{HSD} = 9.1 \times 10^{-5}$) for TLR-2. The occurrence of significant differences for only a subset of the between-groups comparisons for each gene suggested that expression varied non-linearly as PL increased. In particular, we noticed that the standard deviation in MP was greater than in HP and C for all the three significant transcripts, which indicates that increased parasitism could affect not only the mean but also the variance of gene expression, especially for the group of dogs with low-to-moderate infection. In this case, increased variance in MP could lead to loss of power to detect mean differences between groups at small sample sizes. Considering the whole group of infected animals (P = MP + HP), the $2^{-\Delta\Delta Ct}$ method revealed that expression of IL-10, TNF-α and TRL-2 was 1.43, 2.66 and 2.55 fold greater in infected dogs as compared to controls. Moreover, expression of these three genes was highly correlated, with pairwise values of $r_{IL\text{-}10/TNF\text{-}\alpha} = 0.744$ ($p_{cor} = 1.30 \times 10^{-7}$), $r_{IL\text{-}10/TLR\text{-}2} = 0.706$ ($p_{cor} = 1.05 \times 10^{-5}$) and $r_{TNF\text{-}\alpha/TRL\text{-}2} = 0.678$ ($p_{cor} = 3.97 \times 10^{-6}$).

Skin parasitism by *Leishmania* is positively associated with infectiousness to sandflies

We fitted a logistic regression model to assess the contribution of exposure to infected dogs to the probability of sandflies being infected. This analysis was intended to evaluate whether the xenodiagnosis was capable of increasing prevalence of *Leishmania* in exposed sandflies in comparison to control sandflies. We found that the odds of sandfly infection were 4.63 times higher ($p_{logit} = 7.62 \times 10^{-22}$) given exposure to infected dogs as compared to no exposure, with a 95% confidence interval of 3.39 – 6.33. A permutation test for the difference in prevalence between control and tested sandflies was also significant (**Figure 2 – a**), with no larger than observed differences arising by chance in one

million randomizations of the data ($p_{perm} < 10^{-6}$). Given that the xenodiagnosis was successful in shifting the prevalence of *Leishmania* in sandflies, we sought to evaluate the contribution of skin PL to the probability of a sandfly being infected after xenodiagnosis. This analysis revealed that sandflies became significantly ($p_{logit} = 0.009$) more prone to infection as the dog skin PL increased (**Figure 2 – b** and **c**, **Supplementary Figure 2**). Moreover, phlebotomines exposed to HP dogs had a suggestively higher PL than those exposed to MP dogs ($p_{MW} = 0.099$), indicating a potential association between skin and phlebotomine PL.

DISCUSSION

In the present study we found evidence for up-regulation of the immune response genes TLR-2, TNF-α and IL-10 in the skin of dogs naturally affected by CanL. We also found that this up-regulation was positively associated with parasite load. Furthermore, elevated skin parasitism significantly increased infectiousness to phlebotomines, supporting the hypothesis that dogs with higher skin PL have a greater potential to spread the disease.

Increased expression of TLR-2 during canine leishmaniosis has been previously found in several tissues, including skin[28,29], blood[30], liver[29], brain, spleen and lymph node[31]. Here, in addition to a higher relative expression of TLR-2 in affected dogs, we observed a significant difference in transcript abundance between dogs with low-to-moderate and moderate-to-high parasite load. A similar result was observed by Figueiredo et al.[32] in colon samples of dogs naturally infected with *L. infantum*, which suggests a correlation between TLR-2 expression and the progression of infection. Indeed, Monteserrat-Sangrà et al.[30] showed a positive correlation between TLR-2 and PL in unstimulated blood samples from dogs with CanL. When these dogs were treated and showed clinical improvement, TLR-2 transcription was reduced. This is in agreement with findings reported by Murray et al.[22], which showed that blockage of TLR-2 leads to induction of *Leishmania donavani* killing in mice. However, these results are not in concordance with those found by Amorim et al.[16], which reported a higher TLR-2 expression in monocytes associated to a lower parasite load in the skin of naturally infected dogs. Moreover, Turchetti et al.[10] did not observe interference of constitutive transcription of toll-like receptors in intracellular survival of *L. infantum* tested *in vitro*.

Although TLR-2 is deemed to alter production of NO[17,18], our data did not support a correlation with changes in expression of iNOS, in agreement with observations made in TLR-2-deficient mice[22]. Of note, TNF-α has also been previously suggested to modulate induction of iNOS[20,33,34,35], while IL-10 was reported to have inhibitory effects on the production of the enzyme[19,33,36,37]. Since TNF-α (pro-inflammatory) and IL-10 (anti-inflammatory) have putative antagonistic effects on iNOS production, and both transcripts were up-regulated in infected dogs in this study, we speculate whether expression of iNOS in infected dogs has not significantly deviated from control levels as a result of an interplay between TNF-α and IL-10.

We found that TLR-2 expression was positively correlated with TNF-α and IL-10. Gatto et al.[17] observed similar results in human patients with active visceral leishmaniosis. These results indicate that TLR-2 could be regulating TNF-α and IL-10 in an attempt to combat infection. Chandra and Naik[38] also observed an increased level of IL-10 when cells of patients were stimulated with TLR-2 agonists. These data sustain the involvement of TLR-2 in the skin adaptive response to the parasite and the subsequent production of pro-inflammatory and anti-inflammatory cytokines. As a putative downstream effect, the increase in TNF-α expression may help recruiting cells to the infection site. Indeed, an inflammatory infiltrate with predominance of macrophages was observed in the histological

examination of the skin samples (see **Supplementary Figure 3**).

Our xenodiagnosis analysis revealed an increased risk of sandflies becoming infected when exposed to dogs with increased skin parasite load. An association of dog infectiousness to sandflies with high skin parasite numbers tested by qPCR was also found by Courtenay et al.[39] and Borja et al.[40]. Furthermore, a positive correlation of skin parasite load of infected dogs with the number of infected sandflies was also demonstrated by Verçosa et al.[41] and Amorim et al.[16] although parasite load on sandflies was assessed by the presence of amastigotes in the midgut. *A priori*, our result differs from that reported by Laurenti et al.[42], where asymptomatic dogs were found to be more competent in transmitting *L. infantum* to phlebotomines than symptomatic dogs. However, in their morphometric quantitative analysis, they did not find significant differences in the amount of amastigotes/mm^2 of skin between symptomatic and asymptomatic dogs, which imposes a challenge in the comparison of our data with theirs. Nevertheless, our approach of assessing the direct contribution of skin parasite load, as quantified by qPCR, to the probability of a sandfly becoming infected should provide a more robust evaluation of the influence of skin parasitism on the infectiousness to phlebotomines.

Finally, some limitations of our study should be highlighted. First, we evaluated the expression of genes and not the production of cytokines. Second, dogs were naturally infected and, therefore, were at various stages of the disease. Whether or not our results are replicable when cytokines are directly evaluated in dogs with well-characterized disease stages is yet to be clarified. Altogether, our study contributes with further insights on the dynamics of parasite load and immune response in the tissue compartment that serves as the feeding site to the vector and as the first inoculation point in the reservoir of leishmaniosis.

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FIGURE LEGENDS

Figure 1. Dotplots of IL-10, TNF-α and TLR-2 expression (-ΔCt) in the skin of control (C, 0 parasites/mL), moderately parasited (MP, 2.19 x 10^2 to 2.27 x 10^5 parasites/mL) and highly parasited (HP, 2.27 x 10^5 to 4.13 x 10^7 parasites/mL) dogs naturally affected by leishmaniosis. Horizontal black bars represent medians within groups. Significance values were assessed on the basis of a *post hoc* Tukey's Honest Significant Difference (HSD) test.

Figure 2. Xenodiagnosis of field-caught phlebotomines. (**a**) Sandflies subjected to a xenodiagnosis (tested) presented significantly higher prevalence of *Leishmania spp.* than those fed with honey solution (control), as assessed by a permutation test with one million randomizations of the data. (**b**) A logistic regression analysis revealed that prevalence in phlebotomines exposed to infected dogs was significantly (p = 0.009) dependent on the parasite load of the skin. (**c**) Sandflies were more prone to infection when exposed to dogs with increased skin parasite load.

Supplementary Figure 1. Distribution of parasite load values according to dog group. C = eight control uninfected dogs; MP = 11 dogs with parasite load between 2.19 x 10^2 and 2.27 x 10^5 parasites/mL; and HP = 18 dogs with parasite load between 2.27 x 10^5 and 4.13 x 10^7 parasites/mL. Horizontal black bars represent medians within groups.

Supplementary Figure 2. Locally weighed regression (LOESS) of percentage of infected phlebotomines onto parasite load in the dog skin.

Supplementary Figure 3. Histological section of skin of a xenodiagnosed dog with leishmaniosis (stained by HE). (**a**) Diffuse mononuclear infiltrate (lymphocytes and macrophages) expanding the dermis, and presence of multinucleated giant cells (arrow). (**b**) Detail of marked area in (**a**) where it is possible to observe rare neutrophils (arrow) and presence of amastigotes in the cytoplasm of macrophages (*).

Table 1. Primers for the evaluation of the expression of immune genes in the skin

Target	Nucleotides Sequence (5' → 3')	GenBank accession number	Product size (bp)	Reaction efficiency (E)	r^2	Slope	Reference
HPRT-1	F - CACTGGGAAAACAATGCAGA R - ACAAAGTCAGGTTTATAGCCAACA	AY283372	123	105.5	0.986	-3.191	Peters et al.[40]
TLR-2	F - TCGAGAAGAGCCACAAAACC R - CGAAAATGGGAGAAGTCCAG	NM_001005264.2	91	108.8	0.981	-3.127	Mercier et al.[41]
iNOS	F - AGACACACTTCACCACAAGG R - TGCTTGGTGGCGAAGATGAGC	AF077821	285	107	0.848	-3.165	Kaim et al.[42]
IL-10	F- CGACCCAGACATCAAGAACC R - CACAGGGAAGAAATCGGTGA	U33843	101	104.9	0.986	-3.209	Peters et al. [43]
TNF-α	F - ACCCATGTGCTCCTCACC R - AGGGCTCTTGATGGCAGAGA	Z70046	87	100.7	0.946	-3.305	Melo et al.[44]

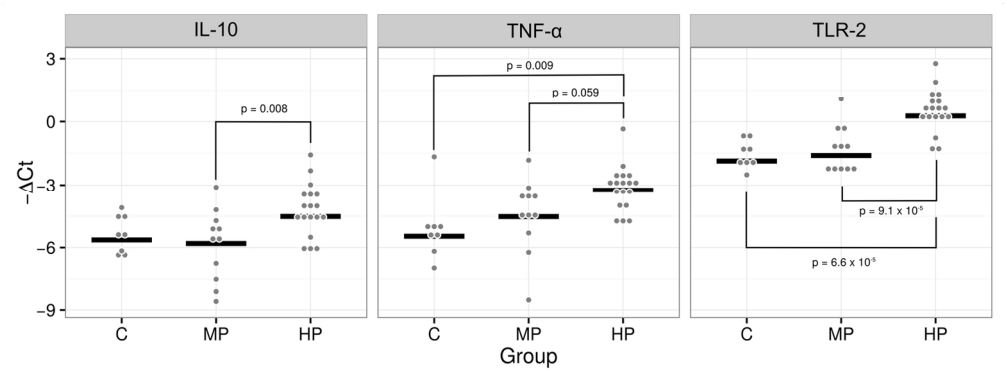
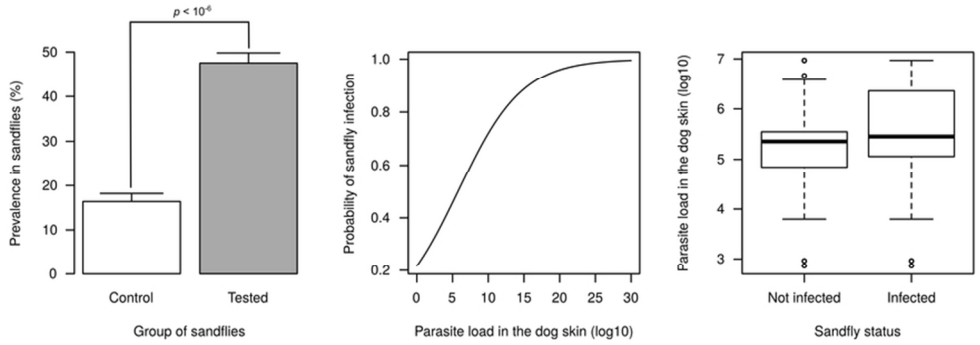


Figure 1. Dotplots of IL-10, TNF-α and TLR-2 expression (-ΔCt) in the skin of control (C, 0 parasites/mL), moderately parasited (MP, 2.19 x 102 to 2.27 x 105 parasites/mL) and highly parasited (HP, 2.27 x 105 to 4.13 x 107 parasites/mL) dogs naturally affected by leishmaniosis. Horizontal black bars represent medians within groups. Significance values were assessed on the basis of a post hoc Tukey's Honest Significant Difference (HSD) test.

76x28mm (600 x 600 DPI)



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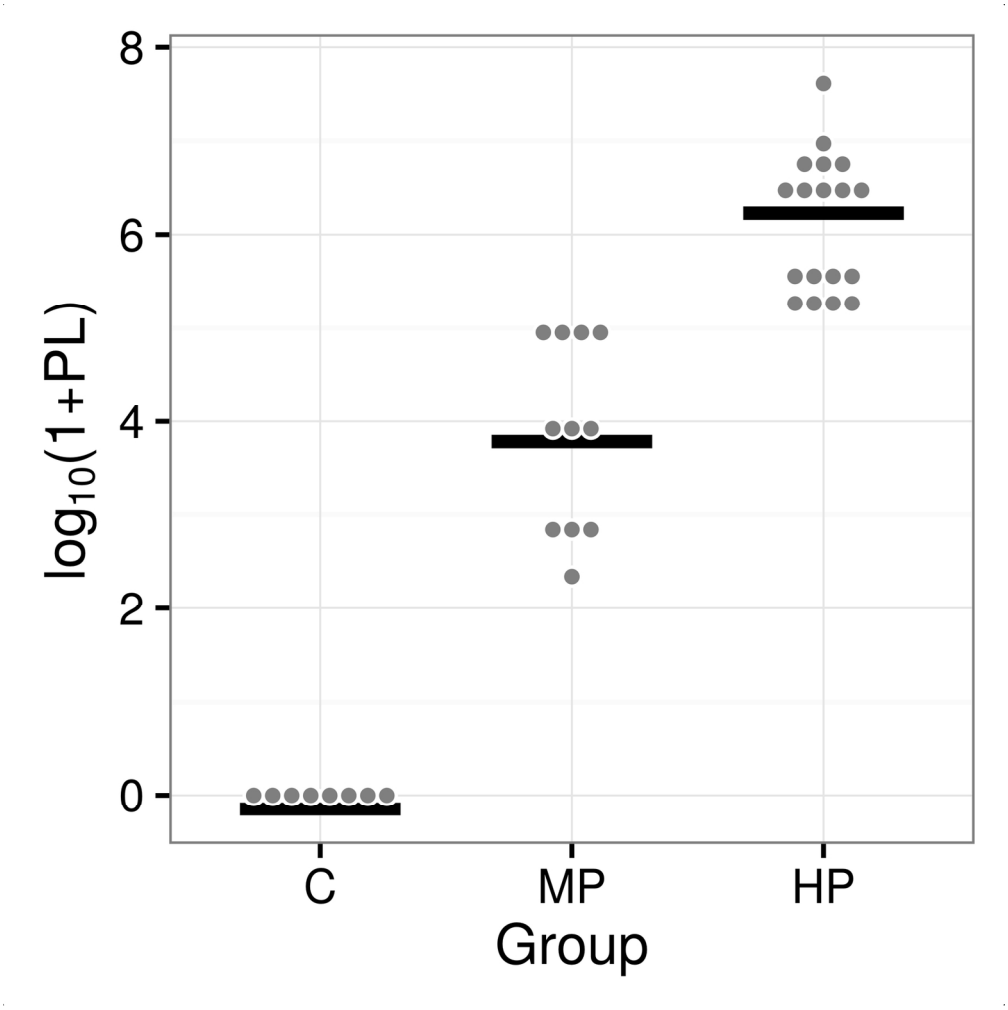
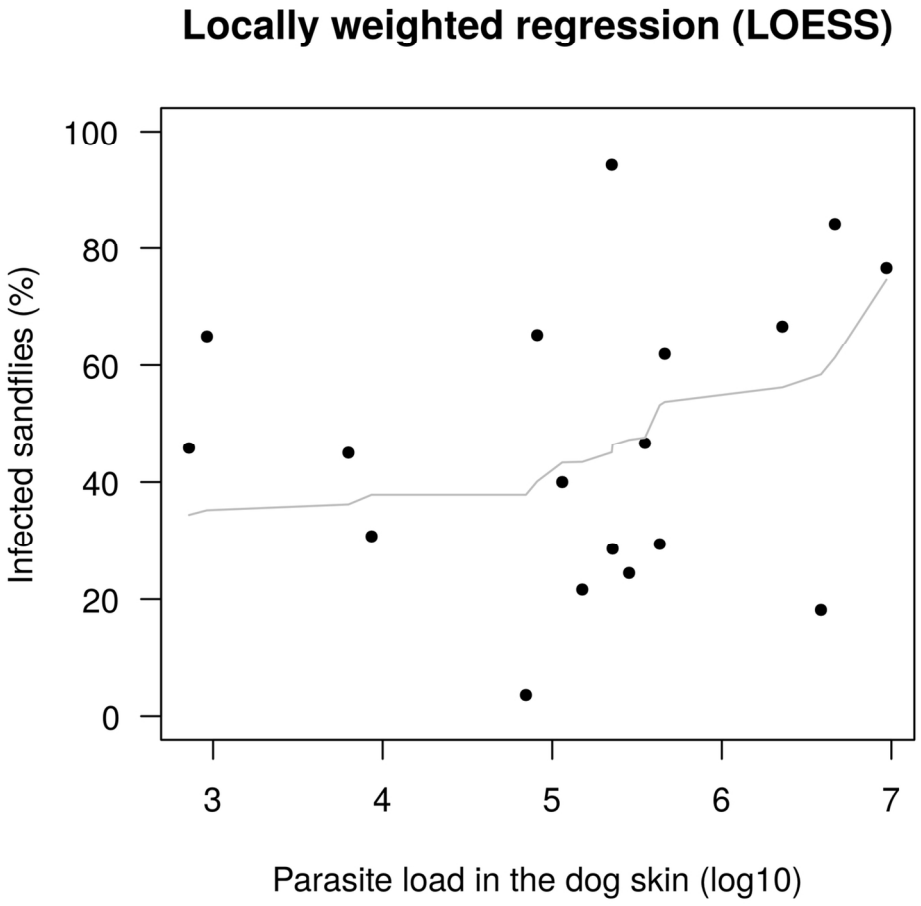


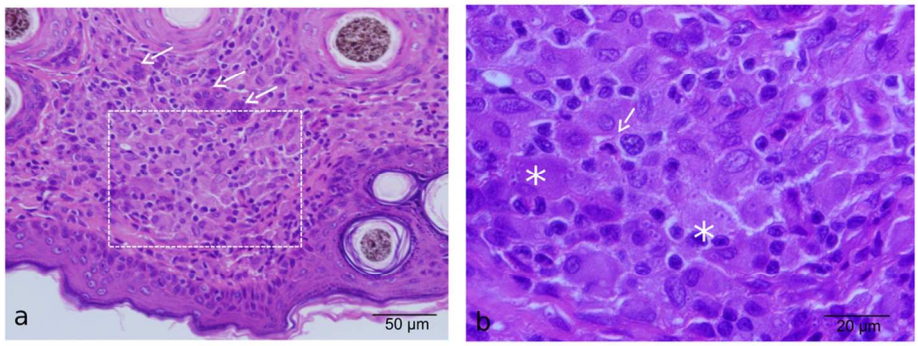
Supplementary Figure 1. Distribution of parasite load values according to dog group. C = eight control uninfected dogs; MP = 11 dogs with parasite load between 2.19 x 102 and 2.27 x 105 parasites/mL; and HP = 18 dogs with parasite load between 2.27 x 105 and 4.13 x 107 parasites/mL. Horizontal black bars represent medians within groups.

76x76mm (600 x 600 DPI)



Supplementary Figure 2. Locally weighed regression (LOESS) of percentage of infected phlebotomines onto parasite load in the dog skin.

127x127mm (300 x 300 DPI)



Supplementary Figure 23. Histological section of skin of a xenodiagnosed dog with leishmaniosis (stained by HE). (a) Diffuse mononuclear infiltrate (lymphocytes and macrophages) expanding the dermis, and presence of multinucleated giant cells (arrow). (b) Detail of marked area in (a) where it is possible to observe rare neutrophils (arrow) and presence of amastigotes in the cytoplasm of macrophages (*).

99x39mm (300 x 300 DPI)